BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Reads on the claimed invention → doesn't read on it because (+) selection but arguably not enabling (but more enabling than application) does not select for deletion

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/00, 15/09, 15/11, 15/13

A1

(11) International Publication Number: WO 94/21787

(43) International Publication Date: 29 September 1994 (29.09.94)

(21) International Application Number: PCT/US94/02676

(22) International Filing Date: 11 March 1994 (11.03.94)

(30) Priority Data:
08/031,802 15 March 1993 (15.03.93) US

(71) Applicant: CELL GENESYS, INC. [US/US]; 322 Lakeside Drive, Foster City, CA 94404 (US).

(72) Inventors: BRENNER, Daniel, G.; 86 Central Avenue, Redwood City, CA 94601 (US). DUBRIDGE, Robert, B.; 2019 Arbor Avenue, Belmont, CA 94002 (US). OTTEN, Gillis, R.; 1183 Foster City Boulevard, Foster City, CA 94404 (US).

(74) Agents: ROWLAND, Bertram, I. et al.; Flehr, Hohbach, Test, Albritton & Herbert, Suite 3400, 4 Embarcadero Center, San Francisco, CA 94111-4187 (US).

(81) Designated States: AU, CA, FI, JP, KR, NO, NZ, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: METHOD FOR DEFINED DELETIONS OF DNA

(57) Abstract

Methods and DNA vectors are provided, which allow for a simplified means to delete large amounts of mammalian chromosomal DNA. The vector comprises a positive selectable marker, negative selectable marker, homologous region for targeting, and a second homologous region for mediating the deletion.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

METHOD FOR DEFINED DELETIONS OF DNA

INTRODUCTION

Technical Field

The field of this invention concerns the genomic modification of vertebrate genes by defined deletions of DNA.

Background

The ability to manipulate a vertebrate genome by homologous recombination has made it possible to alter the genetic makeup of animal cells. When suitable host cells are used, transgenic animal technology can be utilized to permanently change the germline DNA of an entire animal.

The genome can be manipulated to either add or delete DNA sequences. There are a number of uses for animals or cells with targeted deletions of chromosomal DNA. Genes that one does not wish expressed can be removed, and the resulting cells used in therapeutic or experimental settings.

A series of deletions may be made, where a variable amount of DNA is removed by holding one end-point constant, and varying the position of the second end-point. Such a series may be used for the experimental mapping of genes, as there is interest in determining a genetic map for animals, particularly humans.

Some DNA sequences act as negative regulators for gene expression. It may be possible to activate the expression of certain genes by deleting those upstream or downstream elements which are responsible for silencing a

gene using defined deletions of DNA to remove weak promoters or enhancers, and insert stronger foreign promoters or enhancers near the transcriptional initiation site of a native gene. Alternatively, stronger promoters or enhancers may be moved from a wild type gene located upstream near the target gene.

In order to fully utilize this technology there is interest in methods which can create genomic deletions of a defined size, at high frequency, and in certain instances, for creating deletions without the presence of vector DNA sequences being left behind.

Relevant Literature

Scherer and Davis, (1979) Proc. Natl. Acad. Sci. USA **76(10)**:4951-4955, show the replacement of chromosome segments with altered DNA sequences in yeast cells.

Mansour et al. (1988) Nature **336**:348-352, describe a general strategy for targeting mutations to non-selectable genes. Thomas and Capecchi (1987) Cell **51**:503-512, and Thompson et al. (1989) Cell **56(2)**:313-321 describe site-directed mutagenesis by gene targeting to correct the HPRT gene in mouse embryo-derived stem cells.

Methods of targeting genes for modifications in embryonic stem cells are found in Valancius and Smithies (1991) Mol. Cell. Bio. **11**:1402-1408, and Hasty et al. (1991) Nature **350(6315)**:243-246.

Mombaerts et al. (1991) Proc. Natl. Acad. Sci. USA **88(8)**:3084-3087, describe the creation of a large genomic deletion in the T cell antigen receptor beta subunit locus by gene targeting.

SUMMARY OF THE INVENTION

Methods and DNA constructions are provided for simplified defined deletion or mutation of vertebrate chromosomal DNA using homologous recombination. The replacement targeting vector comprises markers for positive and negative selection, homologous regions for

targeting at a specific site, and an additional homologous region to mediate the deletion.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of a targeting construct and target chromosome, as described in the Example, infra.

Figure 2 is a diagram of a bidirectional targeting construct and target chromosome, as described in the Example, infra.

Figure 3 is a diagram of successive stages of deletion, as described infra.

Figure 4 is a diagram of the targeting construct for inactivation of the immunoglobulin kappa light chain J and Constant regions and design of the targeting experiment described in the Example, infra.

Figure 5 is a diagram of the construction of vectors for inactivating the immunoglobulin kappa light chain J and constant regions as described in the Example, infra.

Figure 6 is a diagram of the final deletion vectors for inactivation of the immunoglobulin kappa light chain J and Constant regions as described in the Example, infra.

Figure 7 is an illustration of the Southern analysis of light chain J and Constant region deleted cells as described in the Example, infra.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods utilizing a DNA construct for homologous targeting via replacement of DNA are used to introduce a defined lesion at a specific site in a vertebrate chromosome. By "lesion" is meant an alteration in the DNA sequences at the site. The targeting construct may be used to delete large segments of chromosomal DNA, without leaving vector DNA sequences in the targeted chromosome. The method may also be used to alter a sequence in the target chromosome, without leaving behind vector DNA sequences.

The targeting construct consists of a positive selectable marker, a negative selectable marker, which in

some instances may be the same marker, sequences with homology to the target site in the chromosome, and an additional homologous sequence from a region outside of the sequences to be targeted in the chromosome. Therefore, the target site in the chromosome is defined by the homologous sequences in the vector. The additional homologous DNA sequence, (ADH additional DNA homology) is homologous to a sequence on the same chromosome as the target sequences, which additional sequence can be located either upstream or downstream on the chromosome. The sequence of DNA which lies between the ADH sequence and the target sequences on the chromosome will ultimately be deleted.

A preferred targeting construct for use in the invention is an "omega" or "replacement" targeting vector. However, an "O" or "insertional" targeting vector may also be used in some situations where a single target sequence is sufficient for providing homologous sequences for recombination.

The targeting construct is introduced into a host cell, preferably after linearization, where it undergoes homologous recombination to integrate at the site of the target sequences. The arrangement of elements in the linear construct is shown in Figure 1. However, while an exemplary order of functional units is shown, it should be understood that the order of the functional sequences between the target sequences may be arranged in other ways, depending on the particular use contemplated. In addition, the chromosomal sequences that are to be targeted by the homologous sequences in the targeting construct may be contiguous or separated. Where separated, homologous recombination in a double cross-over event will result in an initial deletion.

For convenience, the DNA elements on the targeting construct that are homologous to DNA sequences in the chromosome will be referred to as vector or "v" homology sequences, while the chromosomal counterparts will be referred to as chromosomal sequences or "c". The two v-

target sequences necessary for homologous recombination will usually lie at the extreme 5′ and 3′ ends of the targeting construct forming arms of homology, except when positive-negative selection is employed in the homologous recombination step, e.g. a negative selectable marker may be a terminal sequence. "c-ADH" or "v-ADH" refers to homologous sequences in the chromosome or vector used for effecting the defined deletion.

A double crossover event at the target sites in the chromosome results in the replacement of the chromosomal region lying between the two c-target sequences, unless the c-target sequences are contiguous, with the construct sequences lying between the two v-target arms of homology. Sequences which are not functional in the vertebrate host, such as bacterial origins of replication and antibiotic resistance, may be outside or inside of the construct arms. Sequences located outside of the arms will not be integrated into the chromosome, while those sequences between the arms will be integrated. The v-ADH region and selectable markers for vertebrate cells will be positioned between the two arms of homology.

After introduction of the construct DNA into the target cell, the positive selectable marker allows one to apply selective pressure, and to grow only those cells which contain the integrated construct. An illustration of the resulting chromosome with integrated construct is shown in Figure 1 as the "Targeted chromosome". The illustration of the "Chromosome," shows the c-target sequences as contiguous, although it should be understood that they may be separated on the chromosome, as described earlier. After integration, one copy of each target sequence and two copies of the ADH sequence will remain. One ADH copy is chromosomal in origin (c-ADH), the other ADH sequence is derived from the targeting construct (v-ADH). The two copies are separated by the chromosomal sequences and at least the negative selectable marker of the targeting construct that lie between the v-ADH and c-ADH sequences.

Once the cells have been positively selected they can be directly negatively selected by the addition of negative selection media to the cells, for example using gancyclovir when the marker is the Herpes thymidine kinase gene. Under negative selection conditions, cells that retain the negative selectable marker positioned between the target sequences, will usually die. Cells that have lost the negative selectable marker because of an intramolecular crossover event involving the homologous c-ADH and v-ADH sequences are able to proliferate under negative selection conditions. Alternatively, the positively selected cells may be screened to identify clones targeted by homologous recombination and the targeted clones can be subjected to negative selection. A second independent stage of negative selection may be needed depending on the absolute efficiencies of the initial intermolecular homologous recombination step and of the deletion resulting from the intramolecular crossover. As noted, the method of the invention may also employ negative selection using a different negative selectable marker from that positioned between the v-target sequences to increase the efficiency of the intermolecular homologous targeting step.

Because the c-ADH and v-ADH sequences are on the same nucleic acid molecule, the deletion is an intramolecular recombination event, and will have a higher efficiency than homologous recombination which is intermolecular. The deletion event contemplated in this invention thus has a much higher probability of providing large deletions of DNA than using intermolecular homologous recombination directly.

An example of the order of elements in the construct is depicted in Figure 1. The 5′ and 3′ ends will usually be bounded by the v-target sequences. The v-ADH sequence and the selectable markers will lie between the v-target sequences. The v-target and v-ADH sequences will all be in the same orientation, that is, the direction of the DNA of the individual components will be the same in the

construct as it is on the chromosome, either 5′ to 3′, or 3′ to 5′.

The negative selectable marker, transcriptionally oriented in either direction, will lie between the v-ADH sequence, and one of the v-target sequences in the construct. The exact position will depend on the position of the c-ADH sequence. On the targeted chromosome, the negative marker lies between the c-ADH and the v-ADH.

The position of the positive selectable marker on the construct will depend on the desired final product, because the excision step may or may not leave behind the positive marker in the chromosomal locus. If the positive and negative selectable markers are both positioned between the c-ADH and the v-ADH in the targeted chromosome, then both will be deleted upon application of negative drug selection. If the negative selectable marker is positioned between the c-ADH and v-ADH - and the positive marker is outside, then the excision step will leave the positive marker behind in the chromosome of the deleted locus. In this situation, it may be preferred to apply both positive and negative selection during the excision step. In general, the positive selectable marker may also be oriented in either transcription direction. As with the positive selectable marker, any exogenous sequences from the construct which do not lie between the c-ADH and the vADH in the targeted chromosome will remain at the locus after the final recombination event.

The arrangement of elements on the chromosome will be as shown in Figure 1. The two c-target sequences will be adjacent to each other to facilitate the initial double crossover event or may be separated to provide for a deletion upon homologous recombination. There will desirably be no more than 20 kb nucleotide distance between the target sequences on the native chromosome, usually not more than about 10 kb, preferably not more than about 1 kb. The distance between the c-ADH and c-target sequences on the chromosome will depend on the desired final product. Normally this distance will be

much larger than the distance between the two c-target sequences. It may be as much as 4000 kb in length, usually not more than about 100 kb in length, and will usually be at least about 500 bp in length.

The length of homologous v-target and v-AnH sequences will be at least about 50 nucleotides in length, preferably at least about 100 nucleotides, usually at least about 0.5 kb in length, and less than about 100 kb in length, usually less than about 20 kb, and preferably less than about 1 kb. The regions of homology will have at least about 90%, preferably at least about 95%, and more preferably at least about 99%, sequence identity with the sequence of the native chromosome.

The v-target sequences may provide for subtle changes in the region of homology, such as introduction of a restriction site, changing the sequence of an enhancer or promoter locus, introducing or removing a splice site, or the like. By subtle change is intended fewer than a total of 10 bp substitutions and/or deletions, where the percentage of base pairs involved in the target sequence does not exceed 10%, usually does not exceed 5%.

Various markers may be used for selection. These include the HPRT minigene (Reid, et al. (1990) Proc. Natl. Acad. Sci. 87:4299-4303), the neo gene for resistance to G418, the HSV thymidine kinase gene for sensitivity to gancyclovir, the hygromycin resistance gene, etc. One may use a marker that can be employed for both positive selection and negative selection, such as HPRT, in which case the marker must be positioned in the construct as a negative selectable marker. Alternatively, one may use separate markers for positive selection, such as neo, hygromycin resistance, etc. and for negative selection, such as HSV-TK, cytosine deaminase, etc.

Other elements of the construct may include sequences which code for specific primer regions which may be used in the polymerase chain reaction (PCR) to identify recombinants, the addition of one or more restriction sites which allow for identification by gel

electrophoresis, the removal of a restriction site at the target locus, or other modification which allows for identification of target cells which have undergone the desired modification.

Various techniques may be used to introduce the linear DNA into the target cell. Techniques include electroporation, calcium precipitated DNA, fusion, transfection, lipofection, and the like. The particular manner in which the DNA is introduced into the cell is not critical to this invention, although electroporation is preferred.

Once the target cells have been transformed, the cells may then be selected by means of the marker gene by plating in a selective medium, growth in selective culture, or Southern analysis. The cells may also be analyzed using PCR by employing primers which will provide for different sized fragments depending upon whether homologous recombination has occurred. In this way, target cells which have undergone the desired modification may be identified.

Modified cells may also be identified by changes in gene expression, if appropriate. If a deletion will remove a negative regulatory element, or silencer, then one would expect increased gene transcription and protein synthesis. Alternatively, if a gene or positive regulatory element, such as an enhancer, is deleted, then one would expect to find loss of transcription and protein synthesis. The presence or absence of the gene product may be detected by using specific antibodies, by functional assays for the gene product, by detecting the absence or presence of mRNA from the gene, or the like. If the gene product is a surface membrane protein, one may use monoclonal antibodies in conjunction with FACS for identification of cells with the desired phenotype.

The subject methodology may by used for mammalian fine-structure genetic analysis, investigation of lesions implicated in genetic diseases and manipulation of gene expression. Genes which may be targeted include beta-

globin, enzymes of erythrocyte metabolism, the complement system, coagulation factors, dystrophin, enzymes of carbohydrate, lipid, amino acid, steroid, purine and pyrimidine metabolism, transport proteins, such as cystic fibrosis transmembrane regulator, immunoglobulin genes, T cell receptor genes, histocompatibility antigens, both major and minor, and the like.

The subject method can find advantage in particular situations where other techniques may not suffice. Because the second step in the process is intramolecular, deletion events may be achievable which could not otherwise be achieved by homologous recombination. Thus, genes may be modified by inactivating loci, where one wishes to inactivate a particular combination of exons, as are present in the immunoglobulin loci, such as the heavy and/or light chain (kappa and lambda) loci, for example to eliminate the ability of a host animal to produce endogenous immunoglobulin heavy and/or light chains.

Another application is the use of the method of the invention to produce "universal donor cells" which do not express surface major histocompatibility complex (MHC) antigens, for transplantation. For example, a portion or substantially all of the MHC can be deleted using the method of the invention. The Class I and Class II MHC antigens are heterodimers, each consisting of an α and a β subunit. In Class I MHC antigens, the β subunit is β_2-microglobulin. Of particular interest is the inactivation of at least one, preferably both, copies of a subunit of an MHC antigen such as β_2-microglobulin. Alternatively, deletion of other genes that affect MHC antigen expression may be accomplished using the method. For example, genes that regulate MHC antigen expression, such as the TAP1, TAP2 genes, LMP2 and LMP7 in the Class II locus that regulate MHC antigen dependent presentation, may be deleted to prevent MHC presentation on mammalian cells.

Depending upon the nature of the mammalian cell, the cell lacking at least one competent MHC antigen may find use as a donor to an allogeneic host or if an embryonic

stem cell, may find use in the production of chimeric mammalian hosts which themselves could be used as a source of organs for transplantation. Of particular interest are methods which provide for cells lacking at least one MHC antigen, Class I or Class II, preferably Class I, which cells may serve a variety of functions in a viable host. The method involves transfection of mammalian cells, particularly normal cells, of a predetermined species with the targeting construct of the invention for deleting DNA in one of the loci related to the β_2-microglobulin gene, the α-subunit(s) of the Class I or II MHC antigens, the β-subunit(s) of the Class II MHC antigens, or the genes associated with regulation of expression of the MHC antigens. The targeting construct will create a deletion resulting in deletion in at least one, usually both copies, of the native gene(s), so as to prevent expression of a functional MHC antigen molecule. When the deletion is made in only one copy of the gene being inactivated, the cells having a single unmutated copy of the target gene are amplified and may be subjected to a second transformation, where the deletion may be the same or different from the first lesion. The resulting transformants are screened for the absence of a functional target antigen and the DNA of the cell may be further screened to ensure the absence of a wild-type target gene. Alternatively, homozygosity as to a phenotype may be achieved by breeding hosts heterozygous for the mutation.

The cells which may be subjected to transformation may be any mammalian cells of interest, which may find use in cell therapy, research, interaction with other cells in vitro or the like. Cells of particular interest include, among other lineages, the islets of Langerhans, adrenal medulla cells which may secrete dopamine, osteoblasts, osteoclasts, epithelial cells, endothelial cells, T-lymphocytes, neurons, glial cells, ganglion cells, retinal cells, embryonic stem cells, liver cells, bone marrow cells, and myoblast (muscle) cells. The cells may be obtained from any mammalian host, including murine and

other rodents, lagomorphs, porcine, feline, bovine, canine, human, etc.

Cells from bare lymphocyte syndrome patients may be isolated in accordance with conventional ways, e.g., panning, affinity columns, magnetic beads, or the like. By employing monoclonal antibodies specific for the lymphoid cell type, B- or T-cell, using monoclonal antibodies for such markers as CD 3, 4, 8, 10, 15 or 19, the desired group of cells and their progenitors may be isolated in a substantially homogeneous composition. The genetically defective cells may be used in the same manner as MHC antigen defective cells produced by homologous recombination.

The MHC antigen deficient cells will be selected to achieve a particular function and be introduced into a mammalian host or used for research or other purpose. Also of interest will be the stem cells which act as the progenitors for any of the above cells, which may be the original progenitor or a progenitor cell which is already dedicated to a particular lineage. Of particular interest will be epidermal cells, such as keratinocytes, retinal epithelial cells, myoblasts, hematopoietic cells, and other cells which may be readily manipulated in vitro, maintained for long periods of time in culture and may be introduced into a host, where the cells will remain viable and functional for long periods of time.

For embryonic stem cells, an embryonic stem cell line may be employed or embryonic stem cells may be obtained freshly from a host such as a murine animal, e.g. a mouse, rat, guinea pig, chinese hamster or other small laboratory animals. The cells may be grown on an appropriate fibroblast-feeder layer or grown in the presence of leukemia inhibiting factor (LIF) and then used for mutation.

The targeting constructs of the invention may be modified to include functional entities that may find use in the preparation of the construct, amplification, transformation of the host cell, and integration of the

construct into the host cell. Techniques which may be used include calcium phosphate/DNA coprecipitates, microinjection of DNA into the nucleus, electroporation, bacterial protoplast fusion with intact cells, transfection, or the like. The DNA may be single or double stranded, linear or circular, relaxed or supercoiled DNA. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology (1990) **185**:527-537. Once the construct has been prepared and manipulated and the undesired sequences removed from the vector, e.g., the undesired bacterial sequences, the DNA construct is now ready to be introduced into the target cells. As already indicated, any convenient technique for introducing the DNA into the target cells may be employed. After transformation of the target cells, many target cells are selected by means of positive and/or negative markers, as previously indicated, neomycin resistance and Acyclovir or Gancyclovir resistance. Those cells which show the desired phenotype may then be further analyzed by restriction analysis, electrophoresis, Southern analysis, polymerase chain reaction or the like. The resulting transformed cells may then be selected by the absence of the target MHC antigen on the surface of the cell. This can be achieved in a variety of ways. For example, one may use antibodies to any epitope of the target MHC antigen in conjunction with complement to kill any cells having the antigen. Alternatively, one may use conjugates of the appropriate antibody, particularly monoclonal antibody with a toxin, such as the A chain of ricin, abrin, diphtheria toxin, or the like. Affinity chromatography may be employed, where antibodies may be used to remove cells comprising the target antigen. The resulting cells which survive should be free of at least one MHC antigen on their surface and now not be as subject to transplant rejection when introduced in vivo as wild-type cells.

The cells may then be grown in an appropriate nutrient medium for expansion and used in a variety of

ways. The cells may be used for transplantation, to become part of an existing tissue, or may be grown to form tissue for transplantation into a non-syngeneic host. For example, with keratinocytes, the cells may be used for replacement of skin in the case of burns, where keratinocytes may be grown to form a continuous layer prior to application. Similarly, the keratinocytes may be used in the case of plastic surgery to replace skin removed from the host for use at another site. Other uses for the keratinocytes include transplantation in decubitus ulcers.

In the case of islets of Langerhans, they may be grown and introduced into capsules or otherwise for insertion into a host for the production of insulin. In the case of retinal epithelial cells, they may be injected into the subretinal space of the eye to treat visual disorders, such as macular degeneration. In the case of immune cells, they may be injected into the bloodstream or elsewhere to treat immune deficiency. In the case of myoblasts, they may be injected at various sites to treat muscle wasting diseases, such as Duchenne muscular dystrophy. For organ transplants, non-syngeneic tissue such as xenogeneic grafts of heart or liver may be performed between related species.

Depending upon the nature of the cells, the therapy involved, and the disorder, the cells may be employed as films, introduced in containers for maintenance at a particular site, or as solid masses impregnated in inert matrices or independent or a matrix. The number of cells administered will vary widely, depending upon the particular application and the manner in which the cells are administered. Administration may be by injection, topical application, incision and placement, in the appropriate location.

Another situation is where one wishes to introduce a sequence at a target site which is refractory to homologous recombination. By employing the subject methodology one can introduce the sequence at a locus

distant from the refractory site and by use of the ADH sequences can delete the region between the site of insertion and the refractory locus, even including the refractory locus. In this manner, one may introduce an enhancer, promoter, exon or the like, into the refractory site, for example to activate the expression of a gene of interest. Thus, the subject methodology can be used to introduce a sequence of interest at a site proximal to a locus to be influenced by the sequence of interest, where the locus is refractory to homologous recombination, and one wishes to delete a region proximal to the refractory locus while bringing an exogenous sequence in proximity to the refractory locus, or the like.

The target cells may be any of a variety of vertebrate cells, particularly animal cells, more particularly mammalian cells. Of particular interest are embryonic stem cells, which may be used to create transgenic animals, and thereby introduce the altered chromosome into the germline of a host animal. Embryonic cells of particular interest include rodent cells, e.g. mouse, rat and guinea pig.

The method may be used to effect deletions in the chromosome of various sizes. Relatively small deletions (500 bp to 15 kb) can be achieved in a defined manner. Such small deletions can also be achieved by standard targeting methodology, by choosing target regions for recombination that are separated by the desired deletion. If standard methodology is used, however, a positive marker must then be tolerated in the final locus. When no extra marker is desired in the final locus, then the method of this invention will permit one to effect a deletion without leaving behind any exogenous sequences.

Large deletions, up to 4000 kb, can be achieved by the methodology of this invention. The size of deletions that can be made by standard methodology are restricted and vary in efficiency. The method of the invention is far more powerful than conventional techniques, because it relies, not on intermolecular homologous recombination for

the excision step, but rather upon intrachromosomal recombination, using negative selection. Large deletion events resulting from the subject invention are more likely to occur at frequencies within the scope of a typical experiment, as compared to a deletion event resulting from a standard targeting experiment.

In certain instances, deletion of large stretches of DNA may delete genes essential to the viability of the cell into which the targeting construct is introduced. In such cases, the essential genes that may be lethal recessive genes, may be added back into the chromosome via the targeting construct or by secondary transfection with other vectors. The essential genes must be added back prior to rendering the deletion homozygous.

The method of the subject invention may have a number of embodiments. Shown in Figure 2 is one embodiment of the invention, wherein a second negative selectable marker and ADH sequence are included in the construct. The second vADH will not normally share any sequence identity with the first v-ADH. The second c-ADH site will be located on the same chromosome as the first c-ADH and the c-target sequences, with the c-target sequences being located between the two c-ADH sequences. After the initial targeting event, negative selection will be applied. This will select for those cells which have deleted the sequence between the first c-ADH and v-ADH. A second round of negative selection, desirably for a different marker, will select for those cells which have deleted the sequence between the second v-ADH and c-ADH. In this way, the deletion will be bidirectional from the site of the initial targeting event.

Alternatively, a series of v-ADH and negative selection marker sequences can be arranged in a sequential manner on the targeting construct to delete in successive stages chromosomal DNA sequences in one direction proceeding from the site of the initial targeting event on the chromosome (Figure 3). Thus, increasingly larger segments of contiguous DNA may be deleted using the method

of the invention. The targeting construct can be constructed with an arrangement of elements as described herein to accomplish bidirectional and staged successive unidirectional deletions to effect particular deletions in the chromosome.

In another embodiment, the ADH sequence may be a repetitive sequence. In that case, the end point for the deletion could be at a number of different sites, where ever the repetitive element is found. In this way, a series of nested deletions could be constructed, with a constant starting point for the deletion, and a variable amount of DNA deleted from that site. Repetitive sequences of interest include the Alu, L1, α-satellite, telomeric or sub-telomeric repeat sequences found in man, and analogous repeat sequences found in other mammals.

The ADH sequence may be homologous to one of the target sequences, but incorporating a genetic lesion or modification. The genetic lesion or mutation may be an insertion, substitution or deletion in the DNA sequence. The number of altered nucleotides will usually be less than about 20 bp, more usually less than about 10 bp, and will be at least 1 bp. Chromosomal sequences will usually not intervene between the ADH and target sequences in this case, therefore the only sequences which are deleted will be vector sequences. In this way, a genetic modification can be introduced without leaving behind marker and vector sequences.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Inactivation of Murine Immunoglobulin Kappa Light Chain J and Constant Regions

A. Design of the targeting experiment

The targeting vector was designed as a replacement type vector initially to delete the constant region as

well as the J region of the kappa locus and replace it with three elements through homologous recombination using regions of homology flanking the constant region (Figure 4). A diphtheria toxin gene (A chain) flanking either or both regions of homology was included in some cases as a negative selectable marker. The three elements consisted of the G418 resistance drug marker, an ADH sequence of mouse DNA homologous to a region of the kappa locus located upstream of the J region, and a thymidine kinase gene. As a result of the inclusion of the ADH sequence in the vector, this initial targeting placed a second copy of the ADH sequence in the locus. This duplication was then used to effect a deletion of the sequences between the segments by applying selective pressure. In this case the cell deletes the thymidine kinase gene that lies between the two segments in order to survive gancyclovir selection.

B. Construction of the targeting vector

The regions of homology were derived from a 129 mouse fetal liver genomic library (Stratagene, San Diego, CA) which was screened using two probes. The first probe was a 1.6 kb HpaI/BamHI fragment (Steinmetz and Zachau, (1980), Nucleic Acids Research 8:1693-1706) that spans the constant region. A lambda phage clone which hybridized to this probe was identified and used to purify and isolate phage DNA. Analysis of this DNA showed that the HpaI/BamHI probe hybridized to a 5.6 kb SphI/BamHI fragment which was then subcloned between the SphI and BamHI sites of the plasmid pUC218 to give the plasmid pUC218/5.6kappa. This subclone contained the J region, an intronic enhancer element and the constant region of the kappa light chain locus. The second probe was a 0.8 kb EcoRI fragment (Van Ness et al. (1981), Cell 27:593-602) that lies 2.8 kb upstream of the J region. Phage DNA from a lambda clone positive for this probe showed that the probe hybridized to a 5.5 kb SacI fragment which was

subcloned into the SacI site of pBluescript SKI (Stratagene) to give the plasmid pSK.5'kappa (Figure 5).

The inactivation vectors which contained a 5' region of homology, a thymidine kinase gene, an ADH, a neomycin resistance gene and a 3' region of homology (Figure 6) flanked in some instances by diphtheria toxin genes were constructed from three plasmids (Figure 5) containing: (a) the 5' fragment of homology with or without the diphtheria toxin gene (DT) driven by the mouse phosphoglycerate kinase gene (PGK) promoter as a negative selectable marker, (b) the herpes thymidine kinase gene (tk) driven by the mouse phosphoglycerate kinase gene (PGK) promoter as a negative selectable marker along with the ADH and the G418 selectable neomycin (neo) gene from pMClNeo (Thomas and Capecchi (1987), Cell 51:503-12), and (c) the 3' fragment of homology with or without the pGK driven DT gene. These three plasmids (Figure 4) were constructed from pSK.A, pSK.B, and pSK.C, respectively, all derived from the plasmid pBluescript SKI by modification of the polylinker. The polylinker of the plasmid pBluescript SKI was modified by cloning between the KpnI and SacI sites a synthetic polylinker defined by the oligonucleotides 5' -GCATATGCCTGAGGGTAAGCATGCGGTAC CGAATTCTATAAGCTTGCGGCCGCAGCT-3' AND 5'-GCGGCCGCAAGCTTAT AGAATTCGGTACCGCATGCTTACCTCAGGCATATGCGTAC-3' to create the plasmid pSK.A, 5'-GAGCTCGGATCCTATCTCGAGGAATTCTATAAGCTTCA TATGTAGCT-3' and 5'-ACATATGAAGCTTATAGAATTCCTCGAGATAGGATC CHAGCTCGTAC-3' to create the plasmid psK.B, 5'-AAGCTTATAG AATTCGGTACCTGGATCCTGAGCTCATAGCGGCCGCAGCT-3' and 5'-GCGGCC GCTATGAGCTCAGGATCCAGGTACCGAATTCTATAAGCTTG TAC-3' to create the plasmid pSK.C.

A diphtheria toxin gene cassette was created in which the gene was flanked by the PGK promoter and the bovine growth hormone polyadenylation signal (Woychik et al. (1984), Proc. Natl. Acad. Sci. U.S.A., 81:3944-3948; Pfarr et al. (1986), DNA 5:115-122). A 2.3 kb XbaI/EcoRI fragment from pTH-1 (Maxwell et al. (1986), Cancer Res. 46:4660-4664) containing the diphtheria toxin A chain

driven by the human metallothionein (hMTII) promoter was cloned into pBluescript SKI cut with XbaI and EcoRI to give the plasmid pSK.DT. The hMTII promoter of pSK.DT was replaced with the PGK promoter from pKJ1 (Tybulewicz et al. (1991), Cell 65:1153-1163). A 0.5 kb XbaI/PstI fragment from pKJ1 was joined to a 3.1 kb XbaI/NcoI fragment from pSK.DT using a PstI/NcoI adapter formed from the oligonucleotides 5'-GGGAAGCCGCCGC-3' and 5'-CATGGCGGC GGCTTCCCTGCA-3' to give the plasmid pSK.pgkDT. A 248 bp fragment containing the bovine growth hormone polyadenylation signal, obtained by PCR amplification of bovine genomic DNA using the oligonucleotide primers 5'-CAGGATCCAGCTGTGCCTTCTAGTTG-3' and 5'-CTGAGCTCTAGACC CATAGAGCCCACCGCA-3', was cloned into pCR1000 (Invitron Corp., San Diego, CA). The polyadenylation sequence was then cloned behind the DT gene as a HindIII/pvuII fragment into pSK.pgkDT cut with HindIII and HpaI to give the plasmid pSK.pgkDTbovGH. The DT gene cassette from pSK.pgkDTbovGH was moved as a 2.1 kb EcoRI/HindIII fragment into pSK.A cut with EcoRI and NotI using a HindIII/NotI adapter formed from the oligonucleotides 5'-AGCTGGAACCCCTTGC-3' and 5'-GGCCGCAAGGGGTTCC-3' to give the plasmid pSK.A/DT. Between the SphI and Bsu365 sites of both pSK.A and pSK.A/DT the 5' region of homology for the kappa locus was cloned. For this purpose a 4.0 kb SphI/Bsu36l fragment resulting from a partial Bsu36I digest followed by a complete SphI digest of plasmid subclone pUC218/5.6kappa was ligated to pSK.A or pSK.A/DT to give the plasmids pSK.A/5'K and pSK.A/DT/5'K, respectively. In the plasmid, pSK.A/DT/5'K, the 5'-end of the DT gene and kappa fragment were adjacent to each other running in the opposite transcriptional orientations.

The PGKtk gene from the plasmid pKJtk (Tybulewicz et al. (1991), Cell 65:1153-1163) was cloned as a 2.7 kb EcoRI/HindIII between the unique EcoRI and HindIII sites of pSK.B to give pSK.B/TK. A 0.8 kb EcoRI fragment used for the ADH was cloned from pSK.5'kappa and was ligated into the EcoRI site of pSK.B/TK to give pSK.B/(TK/0.8K)

such that the 5'-end of the tk gene and kappa fragment were adjacent to each other running in opposite transcriptional orientations. The 1.1 kb neo gene from pMC1Neo was cloned as an XhoI/BamHI fragment between the same sites of pSK.B/(TK/0.8K) to give pSK.B/(TK/0.8K/Neo). The plasmid pSk.C/3'K containing the 3' fragment of homology was constructed by ligating pSK.C digested with BamHI and treated with alkaline phosphatase to the 1.1 kb BgIII/BamHI fragment isolated from pUC218/5.6kappa. In pSK.C/3'K, the kappa fragment was oriented such that transcription proceeded from the SacI in the plasmid polylinker in the direction of the KpnI site. The 2.1 kb DT cassette from pSK.pgkDTbovGH was cloned as an EcoRI/HindIII fragment into the same sites of pSK.C to give pSK.C/3'K/DT.

Three-part ligations were carried out to construct the final targeting plasmids (Figure 6). The 4.0 kb NotI/NdeI fragment from pSK.A/5'K, the 4.8 kb NdeI/SacI fragment from pSK.B/(TK/0.8K/Neo) (obtained by a SacI partial followed by and NdeI digestion of the plasmid), and the 4.0 kb SacI/NotI fragment from pSK.C/3'K were isolated and ligated together to create pK.(TK/0.8K/Neo). The 6.1 kb NotI/NdeI fragment from pSK.A/DT/5'K, the 4.8 kb NdeI/SacI fragment from pSK.B/(TK/0.8K/Neo), and 4.0 kb SacI/NotI fragment from pSK.C/3'K were isolated and ligated together to create pK.DT/(TK/0.8K/Neo). The 6.1 kb NotI/NdeI fragment from pSK.A/DT/5'K, the 4.8 kb NdeI/SacI fragment from pSK.B/(TK/0.8K/Neo), and 6.1 kb SacI/NotI fragment from pSK.C/3'K/DT (obtained by a SacI partial followed by a NotI digestion of the plasmid) were isolated and ligate together to create pK.DT/ (TK/0.8K/ Neo)/DT. For electroporation, the purified plasmid DNAs were first cut with PvuI or ApaLI, then extracted with phenol/chloroform and precipitated by the addition of ethanol before centrifugation. The resultant DNA pellets were resuspended at a concentration of 1 mg/ml in 10 mM Tris-HCl, 1 mM EDTA(TE).

C. Introduction of DNA into cells

The embryonic stem cell line E14-1, a subclone of E14 (Hooper et al., (1987) Nature **326**:292-295), was cultured in DMEM with 4.5 g/l glucose (JRH Biosciences, Irvine, CA) supplemented with 15% heat inactivated fetal calf serum, recombinant murine leukemia inhibitory factor (ESGRO from Gibco BRL, MD, 1000 units/ml), β-mercaptoethanol (0.1 mM), glutamine (2 mM) and penicillin (100 U/ml)/streptomycin (0.1 mg/ml) and grown at 37°C in 5% CO_2. The cells were cultured on mitomycin-treated primary embryonic fibroblast feeder layers essentially as described by Koller and Smithies, (1989), supra. The embryonic fibroblasts were prepared from day 14 embryos carrying the homozygous targeted mutation of β_2-microglobulin (Koller et al., (1990) Science **248**:1227-30). These feeder cells are capable of growth in media containing G418. At 80% confluency, the ES cells were prepared for electroporation by trypsinization, concentration by brief centrifugation and resuspension in HEPES-buffered saline at 2 X 10^7 cells/ml. The cells were equilibrated at room temperature, and DNA (20 μg) linearized (as described above) was added. The mixture was electroporated at 960 μF and 250 V with a BioRad Gene Pulser. The cells were left to stand at room temperature for 10 minutes before plating onto a 4 x 10 plate of mitomycin C treated feeders (3 x 10^6 feeder cells/plate). After incubation at 37°C for 48 hr, the cells were fed media containing G418 at 150 μg/ml (effective concentration).

D. Analysis of constant region-targeted ES cells

After 7-10 days under drug selection with G418, the individual surviving colonies were each picked and dissociated in a drop of trypsin in a 96-well plate and then incubated at 37°C for 2 min. The cells from each colony were transferred into a well of a 24-well plate containing mitomycin C treated feeder cells and selective media with G418 at 150 μg/ml. After an additional 5-8 days, 20% of the cells in each well were frozen and the

remainder were used to prepare genomic DNA. The cells were lysed with 0.4 ml of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 1% SDS, and proteinase K (1 mg/ml) by incubation overnight at 50°C. The DNA was purified by phenol extraction and ethanol precipitation. The DNA pellets were washed with 70% ethanol, dried and resuspended in TE (20 μl).

Southern analysis was carried out using Bgl II digested genomic DNA from each sample. A 2.3 kb fragment was detected from the native ES cell locus, while a larger 4.9 kb fragment was detected from a targeted ES cell locus (Figure 7), using as a probe the 1.2 kb BamHI/Bgl II fragment isolated from the original phage DNA contiguous with the fragment used for the 3′ homology in the targeting vector. The fragment increased in size because the Bgl II site in the Bgl II/BamHI fragment was lost in the targeting plasmid due to the joining of a Bgl II site to a BamHI site in the ligation, and a new Bgl II site located in the thymidine kinase gene was introduced into the targeted locus.

From a screen by the Southern analysis described above, of a total of 103 clones derived from experiments using three different targeting plasmids, 5 cell lines were identified which carried the intended mutation (Table 1).

Table 1

C_K Light Chain Targeting Result in E14-1 Cells

Construct	Number Screened by Southern	Number of Confirmed Targeted Clones	Clone Designation	Frequency of Targeting
pK.(TK/0.8K/Neo)	44	2	625,691	1/22
pK.DT(TK/0.8/Neo)	42	2	604,611	1/21
pK.DT(TK/0.8K/Neo) DT	17	1	653	1/17

Further analysis of genomic DNA produced from 4 of the positive clones (clones 625, 604, 611 and 653) after being thawed and expanded, re-confirmed the initial observations. Using a second probe, a 1.7 kb HindIII/Bgl II fragment which spanned the J region of the kappa locus, the correct integration pattern was checked for homologous targeting at the 5′ end of the targeting vector. Thus, using this probe with an EcoRI digest of the genomic DNA, a 15 kb fragment was detected from the unmodified allele. In contrast, a 7.8 kb fragment from the targeted allele was observed as a result of the introduction of a new EcoRI site in the thymidine kinase gene during the homologous integration (Figure 7).

E. In vitro excision of J region DNA from targeted clones

In order to effect the desired deletion from the homologously targeted kappa locus, cells from clone 653 were plated on feeder cells at a density of 0.5-1 x 10^6 cells/10 cm dish in the presence of both gancyclovir (2 μM) and G418 (150 μg/ml). After growth for 5 days in the presence of both drugs, clones were picked as described above into 24-well plates and grown under G418 selection alone. After an additional 5-8 days, 20% of the cells in each well were frozen and the remainder used to prepare genomic DNA as previously described.

F. Analysis of J/constant region deleted ES cells

Southern analysis was carried out using BamHI digested genomic DNA from each sample. Using as a probe the 0.8 kb EcoRI fragment used as the ADH in the targeting vectors, as 12.7 kb fragment was detected from the native ES cell locus, while a larger 15.8 kb fragment was detected from the constant region-targeted ES cell locus (Figure 7) using DNA from clone 653. The fragment increased in size because of the insertion of the tk gene, the ADH, and the neo gene into the 12.7 kb BamHI fragment. There was also a new BamHI site introduced at the 3′ end

of the neo gene. Using DNA from the J/constant region deleted cells, a 5.5 kb fragment was detected from the modified locus in addition to the 12.7 kb fragment from the untargeted allele as predicted from analysis of the restriction map. From this screen by Southern analysis of 2 clones produced from 1.5 x 10^6 ES cells plated (clone 653), one cell line (clone 653B) was identified which carried the intended deletion of the J and constant regions.

Further analysis of genomic DNA produced from clone 653B after being thawed and expanded re-confirmed the initial observations. Using the 0.8 kb EcoRI fragment, the deletion was checked with two other restriction digests which should cut outside of the excised region on the 5′ and 3′ ends of the targeting vector. Thus using this probe with a Bgl II digest of the genomic DNA from the unexcised clone 653, a 2.6 kb fragment was detected from both the unmodified and modified alleles, whereas an additional 4.9 kb fragment was observed from the targeted allele only (Figure 7). This 4.9 kb fragment was the same as that detected with the 1.2 kb BamHI/Bgl II fragment used previously. Using DNA from clone 653B, a BgIII digest revealed a 5.8 kb fragment in addition to the 2.6 kb fragment from the unmodified allele. A SacI digest of clone 653 DNA probed with the 0.8 kb EcoRI fragment showed a 5.5 kb fragment from both the unmodified and modified alleles and a 3.1 kb fragment from the targeted allele only (Figure 7). The 5.5 kb fragment was also detected in DNA from clone 653B and an additional 2.0 kb fragment. The 5.8 kb Bgl II fragment and the 2.0 kb SacI fragment were consistent with an analysis of the predicted restriction map for a precise excision step in which 10.3 kb of DNA were deleted including the J region, the tk gene, and one copy of the ADH.

G. Generation of Germline Chimeras

The unmodified E14-1 cells contributed to the germline at a high frequency after injection into C57BL/6J

blastocysts. The cells from the targeted ES cell line 653B which were grown on primary feeder layers as described, were trypsinized and resuspended in injection medium, which consisted of DMEM supplemented with 15% fetal calf serum, 20 mM HEPES (pH 7.3), antibiotics and β-mercaptoethanol. The ES cells (10-15) were injected into each blastocyst, and injected blastocysts (10) were transferred to a pseudopregnant female mouse, 5 into each uterine horn. Chimeric pups are identified by chimeric coat color. Chimeric males are bred to C57BL/6J females, and germline transmission of the 129 derived ES cell (unmodified or targeted) is detected by the agouti coat color of the F1 offspring.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Brenner, Daniel G.
Dubridge, Robert B.
Otten, Gillis R.

(ii) TITLE OF INVENTION: Method for Defined Deletions of DNA

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Flehr, Hohbach, Test, Albritton & Herbert
(B) STREET: Four Embarcadero Center, Suite 3400
(C) CITY: San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94111

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US94/
(B) FILING DATE: 11-MAR-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Rowland, Bertram I.
(B) REGISTRATION NUMBER: 20,015
(C) REFERENCE/DOCKET NUMBER: FP-57537/BIR CELL-015

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 494-8700
(B) TELEFAX: (415) 494-8771
(C) TELEX: 910 277299

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..53)
(D) OTHER INFORMATION: /note= "Complementary to SEQ ID NO:2, bases 1 to 52."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (2..7)
(D) OTHER INFORMATION: /note= "NdeI restriciton site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (8..14)

(D) OTHER INFORMATION: /note= "Bsu36I restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (19..24)
(D) OTHER INFORMATION: /note= "SphI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (25..30)
(D) OTHER INFORMATION: /note= "KpnI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (31..36)
(D) OTHER INFORMATION: /note= "EcoRI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (40..45)
(D) OTHER INFORMATION: /note= "HindIII restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (46..53)
(D) OTHER INFORMATION: /note= "NotI restriction site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCATATGCCT GAGGGTAAGC ATGCGGTACC GAATTCTATA AGCTTGCGGC CGCAGCT 57

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..52)
(D) OTHER INFORMATION: /note= "Complementary to SEQ ID NO:1, bases 1 to 53."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..8)
(D) OTHER INFORMATION: /note= "NotI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (9..14)
(D) OTHER INFORMATION: /note= "HindIII restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (18..23)
(D) OTHER INFORMATION: /note= "EcoRI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (24..29)
(D) OTHER INFORMATION: /note= "KpnI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (30..35)
(D) OTHER INFORMATION: /note= "SphI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (39..45)
(D) OTHER INFORMATION: /note= "Bsu36I restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (46..51)
(D) OTHER INFORMATION: /note= "NdeI restriction site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCGGCCGCAA GCTTATAGAA TTCGGTACCG CATGCTTACC TCAGGCATAT GCGTAC 56

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..43)
(D) OTHER INFORMATION: /note= "Complmentary to SEQ ID NO:4, bases 1 to 43."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..6)
(D) OTHER INFORMATION: /note= "SacI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (7..12)
(D) OTHER INFORMATION: /note= "BamHI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (16..21)
(D) OTHER INFORMATION: /note= "XhoI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (22..27)
(D) OTHER INFORMATION: /note= "EcoRI restriction stie."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (31..36)
(D) OTHER INFORMATION: /note= "HindIII restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (37..42)
(D) OTHER INFORMATION: /note= "NdeI restriction site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGCTCGGAT CCTATCTCGA GGAATTCTAT AAGCTTCATA TGTAGCT 47

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..43)
(D) OTHER INFORMATION: /note= "Complmentary to SEQ ID NO:03, bases 1 to 43."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (2..7)
(D) OTHER INFORMATION: /note= "NdeI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (8..13)
(D) OTHER INFORMATION: /note= "HindIII restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (17..22)
(D) OTHER INFORMATION: /note= "EcoRI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (23..28)
(D) OTHER INFORMATION: /note= "XhoI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (32..37)
(D) OTHER INFORMATION: /note= "BamHI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (38..43)
(D) OTHER INFORMATION: /note= "SacI restriction site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACATATGAAG CTTATAGAAT TCCTCGAGAT AGGATCCGAG CTCGTAC 47

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..46)
(D) OTHER INFORMATION: /note= "Complementary to SEQ ID NO:6, bases 1 to 46."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..6)
(D) OTHER INFORMATION: /note= "HindIII restriction stie."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (10..15)
(D) OTHER INFORMATION: /note= "EcoRI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (16..21)
(D) OTHER INFORMATION: /note= "KpnI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (23..28)
(D) OTHER INFORMATION: /note= "BamHI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (30..35)
(D) OTHER INFORMATION: /note= "SacI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (39..46)
(D) OTHER INFORMATION: /note= "NotI restriction site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAGCTTATAG AATTCGGTAC CTGGATCCTG AGCTCATAGC GGCCGCAGCT 50

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..46)
(D) OTHER INFORMATION: /note= "Complementary to SEQ ID NO:5, bases 1 to 46."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..8)
(D) OTHER INFORMATION: /note= "NotI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (12..17)
(D) OTHER INFORMATION: /note= "SacI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (19..24)
(D) OTHER INFORMATION: /note= "BamHI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (26..31)
(D) OTHER INFORMATION: /note= "KpnI restriction site."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (32..37)
(D) OTHER INFORMATION: /note= "EcoRI restriction stie."

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (41..46)
(D) OTHER INFORMATION: /note= "HindIII restriction site."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGGCCGCTA TGAGCTCAGG ATCCAGGTAC CGAATTCTAT AAGCTTGTAC 50

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (1..13)
(D) OTHER INFORMATION: /note= "Complmentary to SEQ ID NO:8, bases 5 to 17."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAAGCCGC CGC 13

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (5..17)
(D) OTHER INFORMATION: /note= "Complementary to SEQW ID NO:7, bases 1 to 13."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CATGGCGGCG GCTTCCCTGC A 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAGGATCCAG CTGTGCCTTC TAGTTG 26

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTGAGCTCTA GACCCATAGA GCCCACCGCA 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (5..16)
(D) OTHER INFORMATION: /note= "Complementary to SEQ ID NO:12, bases 5 to 16."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTGGAACC CCTTGC 16

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: complement (5..16)
(D) OTHER INFORMATION: /note= "Complementary to SEQ ID NO:11, bases 5 to 16."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGCCGCAAGG GGTTCC 16

WHAT IS CLAIMED IS:

1. A method of introducing a defined deletion at a chromosomal target site in a host cell, said method comprising:

(1) transforming viable cells with a DNA targeting construct, said construct comprising:

(A) two target DNA sequences, said target sequences being homologous to DNA sequences defining said target site, separated by:

(B) a first additional DNA homology (v-ADH) sequence, said first v-ADH sequence being homologous to a first chromosomal ADH sequence (c-ADH) at a site located at a distance from said target site on the same chromosome, and

(C) at least one selectable marker;

(2) selecting for transformed cells that express said selectable marker;

(3) selecting for transformed cells that lack said selectable marker;

whereby homologous recombination occurs at said target site, resulting in integration of said DNA construct, and an intramolecular recombination occurs between said first v-ADH sequence and said first c-ADH sequence, resulting in a chromosomal deletion between said target site and said first c-ADH site.

2. A method according to Claim 1, wherein said at least one selectable marker comprises one marker for positive selection and a different marker for negative selection.

3. A method according to Claim 2, wherein said marker for positive selection is positioned on said DNA construct between said marker for negative selection and said v-ADH sequence.

4. A method according to Claim 2, wherein said ADH sequence comprises a mammalian repetitive DNA sequence.

5. A method according to Claim 1, wherein said DNA construct further comprises a second v-ADH sequence, and a second marker for negative selection, said second v-ADH sequence being homologous to a c-ADH sequence at a second c-ADH site located on the same chromosome as said target site, said target site being located between said first c-ADH site and said second c-ADH site on said chromosome.

6. A method according to Claim 2, wherein said DNA construct further comprises (1 + x) v-ADH additional vADH sequences, and (1 + x) markers for negative selection, said (1 + x) v-ADH sequences being homologous to (1 + x) additional c-ADH sequences at (1 + x) additional c-ADH sites located in series, each c-ADH sequence adjacent the next in the series, on one side of said target site on the same chromosome, wherein "x" is any number of additional DNA homology sequences, whereby successive deletions of chromosomal DNA adjacent to the target site are accomplished.

7. A method according to Claim 2, wherein said distance between said target site and said c-ADH site is from about 500 bp to about 4000 kb in length.

8. A method according to Claim 7, wherein included in the chromosomal sequence between said target site and said v-ADH sequence is a DNA sequence encoding a transcriptional enhancer and/or silencer.

9. A method according to Claim 2, wherein said vADH sequence is homologous to one of said target sequences, and differs by at least 1 bp.

10. A method according to Claim 1, wherein said marker is selected from the group consisting of the HPRT minigene, the neo gene, the HSV thymidine kinase gene, hygromycin resistance, and HPRT.

11. A method of introducing a deletion at a mammalian immunoglobulin locus in a host cell, said method comprising:

(1) transforming viable mammalian cells with a DNA construct, said construct comprising:

(A) two target DNA sequences, said target sequences being homologous to DNA sequences at the constant region of said mammalian immunoglobulin locus,

(B) said target sequences being separated by a v-ADH sequence, said ADH sequence being homologous to a c-ADH DNA sequence at site located at a distance 5′ or 3′ to said target site on the same chromosome, and

(C) a positive selectable marker, and a

(D) negative selectable marker,

(2) selecting for transformed cells which express said positive selectable marker;

(3) selecting for transformed cells which lack said negative selectable marker;

whereby homologous recombination occurs at said mammalian immunoglobulin locus, resulting in integration of said DNA construct, and an intramolecular recombination occurs between said v-ADH and c-ADH sequence in said chromosome, resulting in a chromosomal deletion between said target site and said c-ADH site.

12. A method according to Claim 11, wherein said immunoglobulin locus is selected from the group consisting of the immunoglobulin heavy and light chain loci.

13. A method according to Claim 11, wherein said immunoglobulin locus is a light chain locus.

14. A method according to Claim 13, wherein said immunoglobulin locus is a kappa chain locus.

15. A method according to Claim 13, wherein said immunoglobulin locus is a lambda chain locus.

16. A DNA construct comprising:

(A) two target DNA sequences, said target sequences being homologous to proximal DNA sequences at a target site of a chromosome; said target sequences separated by a

(B) v-ADH sequence, said v-ADH sequence being homologous to a c-ADH sequence at a chromosomal site located distal from said target homologous DNA sequences on the same chromosome; and

(C) a marker for positive selection; and

(D) a marker for negative selection.

17. A DNA construct according to Claim 16, wherein said v-ADH sequence comprises a mammalian repetitive DNA sequence.

18. A DNA construct according to Claim 16, wherein said construct further comprises a second DNA v-ADH sequence, said second v-ADH sequence being homologous to a c-ADH sequence at a second chromosomal site located on the same chromosome as said target site, said target site being located between said first c-ADH site and said second c-ADH site on said chromosome, and a second marker for negative selection.

19. A DNA construct according to Claim 16, wherein the chromosomal sequence between said target site and said c-ADH sequence is from about 500 bp to about 4000 kb in length.

20. A DNA construct according to Claim 16, wherein the chromosomal sequence between said target site and said first v-ADH sequence further comprises

DNA sequences encoding a transcriptional enhancer and/or silencer.

21. A construct according to Claim 16, wherein said marker is selected from the group consisting of the HPRT

minigene, the neo gene, the HSV thymidine kinase gene, hygromycin resistance, and HPRT.

22. A method for inactivating a target locus located on a chromosome between a target site and a chromosomal c-ADH DNA sequence at a distance from said target site on the same chromosome comprising:

1) transforming viable mammalian cells with a DNA construct, said construct comprising:

(A) two target DNA sequences, said target sequences being homologous to DNA sequences at a target site on a chromosome; said target sequences separated by a

(B) v-ADH sequence, said v-ADH sequence being homologous to a c-ADH sequence at a chromosomal site located distal from said target site on the same chromosome;

(C) a marker for positive selection; and

(D) a marker for negative selection,

(2) selecting for transformed cells that express said positive marker;

(3) selecting for transformed cells that lack said negative marker;

whereby homologous recombination occurs at said target site, resulting in integration of said DNA construct, and an intramolecular recombination occurs between said v-ADH sequence and said c-ADH sequence, resulting in a chromosomal deletion of at least a portion of said target locus between said target site and said cADH site, wherein said target locus is inactivated.

23. A method according to Claim 22, wherein said target locus is the MHC.

24. A method according to Claim 22, wherein said target locus is the β_2-microglobulin subunit locus.

25. A method for producing mammalian donor cells lacking MHC Class I and/or Class II antigens for use in transplantation comprising:

(1) transforming viable mammalian cells with a DNA construct for inactivating Class I and/or Class II MHC antigens, said construct comprising:

(A) two target DNA sequences, said target sequences being homologous to DNA sequences within the MHC,

(B) said target sequences being separated by a v-ADH sequence, said ADH sequence being homologous to a c-ADH DNA sequence at site located at a distance 5′ or 3′ to said MIIC on the same chromosome, and

(C) a positive selectable marker, and a

(D) negative selectable marker,

(2) selecting for transformed cells which express said positive selectable marker;

(3) selecting for transformed cells which lack said negative selectable marker;

whereby homologous recombination occurs within said MHC, resulting in integration of said DNA construct, and an intramolecular recombination occurs between said v-ADH and c-ADH sequence in said chromosome, resulting in a chromosomal deletion between said target site and said cADH site inactivating Class I and/or Class II MHC antigens.

26. A method according to Claim 25, wherein said chromosomal deletion comprises the β_2-microglobulin locus.

27. A mammalian cell that does not express surface MHC antigen produced by the method according to Claim 25.

28. A mammalian cell according to Claim 27, wherein said cell is murine.

29. A mammalian cell according to Claim 27, wherein said cell is human.

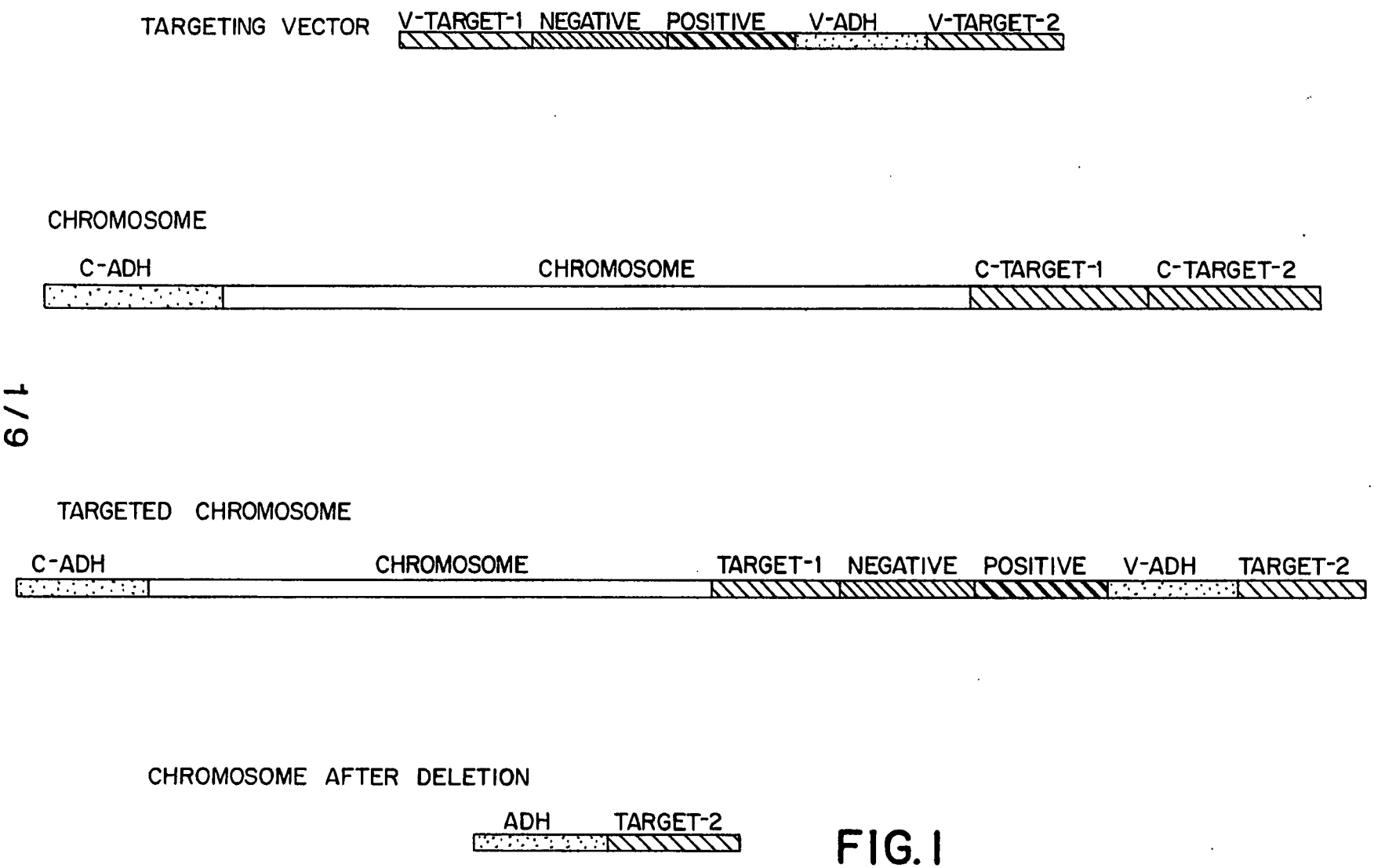

TARGETING VECTOR
V-TARGET-1
NEGATIVE
POSITIVE
V-ADH
V-TARGET-2
CHROMOSOME
C-ADH
CHROMOSOME
C-TARGET-1
C-TARGET-2
TARGETED CHROMOSOME
C-ADH
CHROMOSOME
TARGET-1
NEGATIVE
POSITIVE
V-ADH
TARGET-2
CHROMOSOME AFTER DELETION
ADH
TARGET-2

FIG. 1

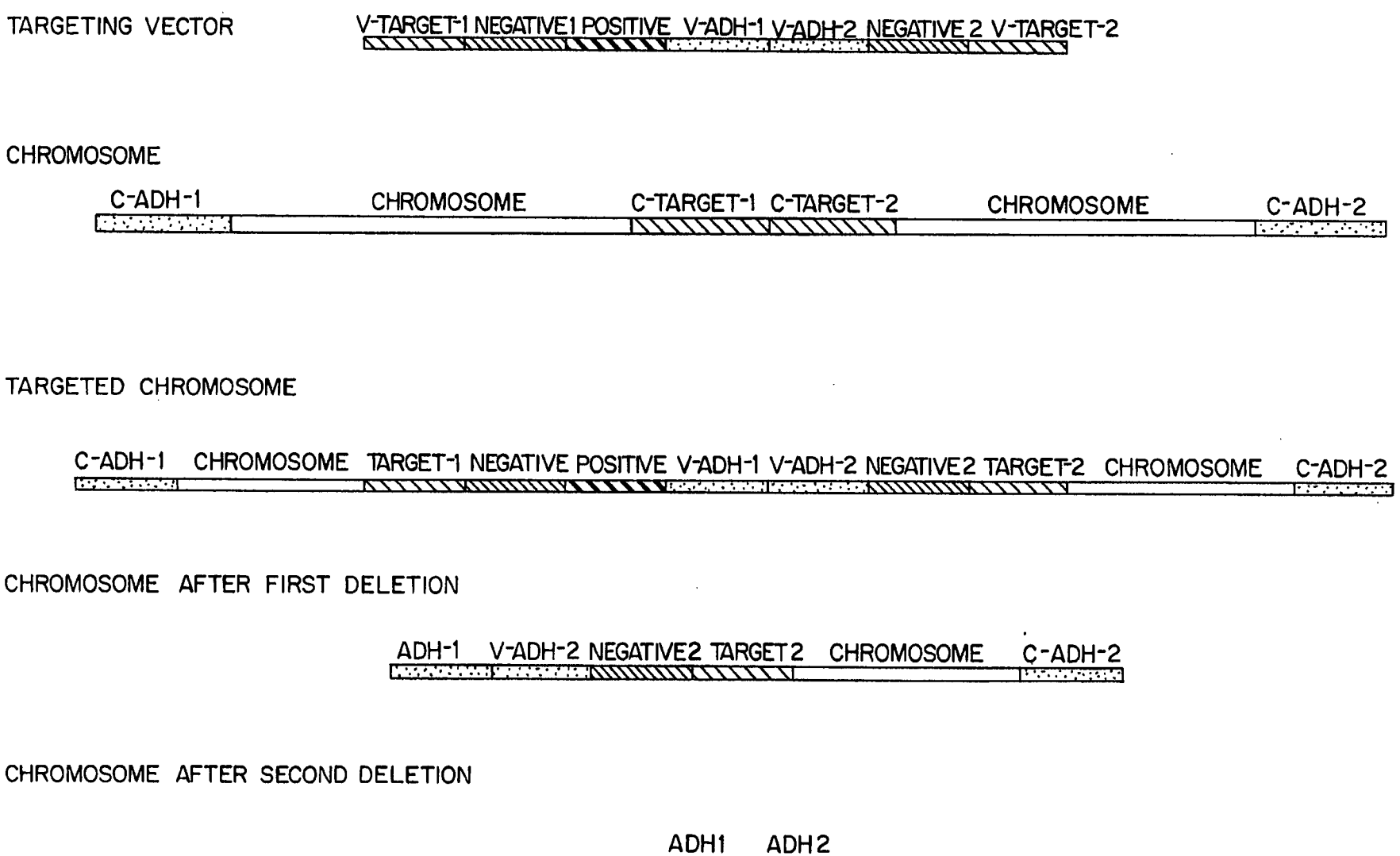

TARGETING VECTOR
V-TARGET-1 NEGATIVE1 POSITIVE V-ADH-1 V-ADH-2 NEGATIVE 2 V-TARGET-2
CHROMOSOME
C-ADH-1
CHROMOSOME
C-TARGET-1 C-TARGET-2
CHROMOSOME
C-ADH-2
TARGETED CHROMOSOME
C-ADH-1 CHROMOSOME TARGET-1 NEGATIVE POSITIVE V-ADH-1 V-ADH-2 NEGATIVE2 TARGET-2 CHROMOSOME C-ADH-2
CHROMOSOME AFTER FIRST DELETION
ADH-1 V-ADH-2 NEGATIVE2 TARGET2 CHROMOSOME C-ADH-2
CHROMOSOME AFTER SECOND DELETION
ADH1 ADH2

FIG.2

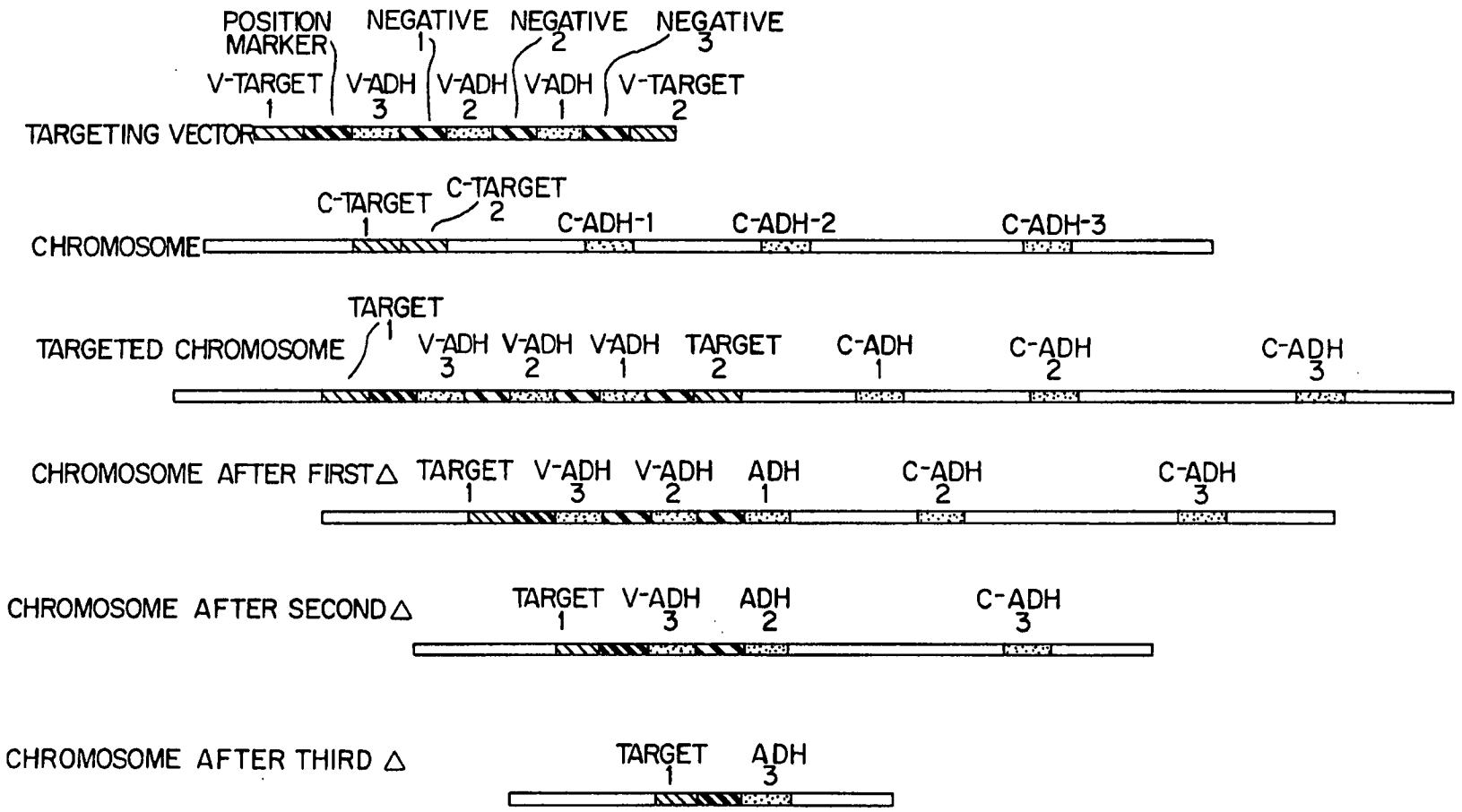

POSITION MARKER
NEGATIVE 1
NEGATIVE 2
NEGATIVE 3
V-TARGET 1
V-ADH 3
V-ADH 2
V-ADH 1
V-TARGET 2
TARGETING VECTOR
C-TARGET 1
C-TARGET 2
C-ADH-1
C-ADH-2
C-ADH-3
CHROMOSOME
TARGET 1
TARGETED CHROMOSOME
V-ADH 3
V-ADH 2
V-ADH 1
TARGET 2
C-ADH 1
C-ADH 2
C-ADH 3
CHROMOSOME AFTER FIRST Δ
TARGET 1
V-ADH 3
V-ADH 2
ADH 1
C-ADH 2
C-ADH 3
CHROMOSOME AFTER SECOND Δ
TARGET 1
V-ADH 3
ADH 2
C-ADH 3
CHROMOSOME AFTER THIRD Δ
TARGET 1
ADH 3

FIG. 3

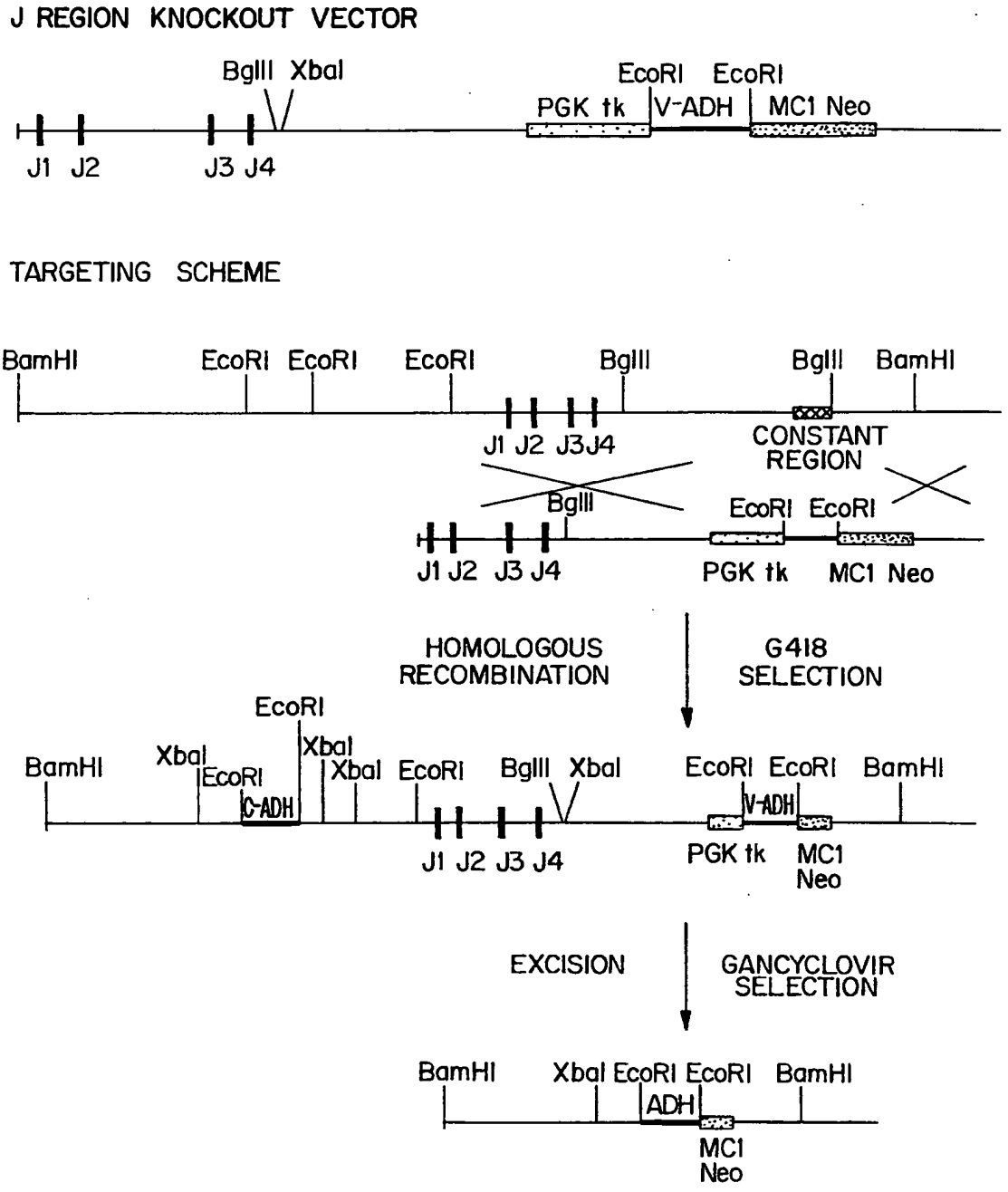

J REGION KNOCKOUT VECTOR
BglII XbaI
EcoRI EcoRI
PGK tk
V-ADH
MC1 Neo
J1 J2
J3 J4
TARGETING SCHEME
BamHI
EcoRI EcoRI
EcoRI
BglII
BglII BamHI
J1 J2 J3J4
CONSTANT REGION
BglII
EcoRI EcoRI
J1 J2 J3 J4
PGK tk
MC1 Neo
HOMOLOGOUS RECOMBINATION
G418 SELECTION
EcoRI
BamHI
XbaI
XbaI
EcoRI
C-ADH
XbaI
EcoRI
BglII XbaI
EcoRI EcoRI
BamHI
V-ADH
J1 J2 J3 J4
PGK tk
MC1 Neo
EXCISION
GANCYCLOVIR SELECTION
BamHI
XbaI EcoRI EcoRI
BamHI
ADH
MC1 Neo

FIG.4

NdeI BamHI SphI KpnI EcoRI HindIII NotI

GCATATGCCTGACGTAAGCATGCGGTACCGAATTCTATAAGCTTGCGGCCGCAGCT
CATGCGTATACGGACTGCATTCGTACGCCATGGCTTAAGATATTCGAACGCCGGCG

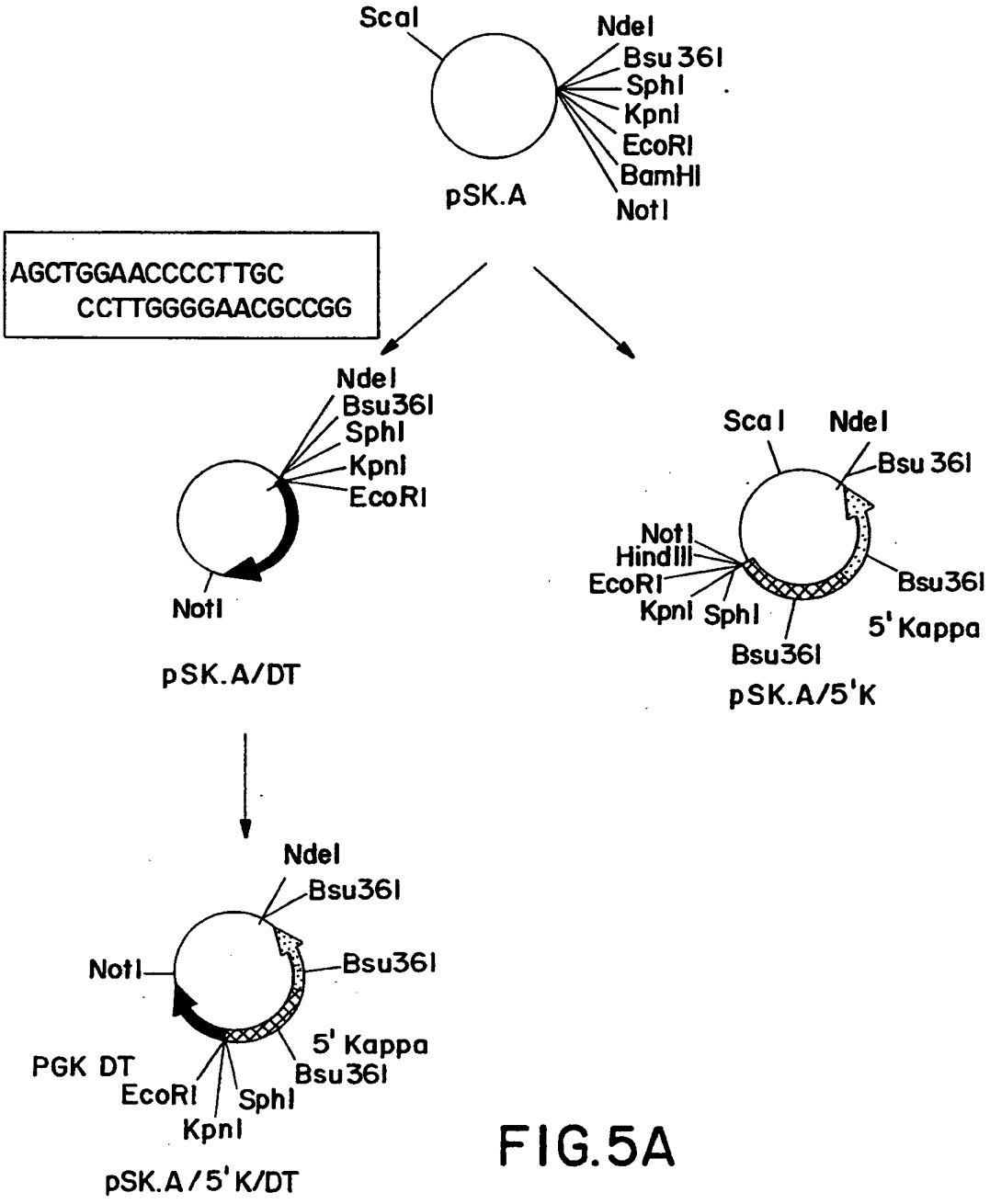


FIG.5A

SacI BamHI XhoI EcoRI HindIII NdeI
GAGCTCGGATCCTATCTCGAGGAATTCTATAAGCTTCATATGTAGCT
CATGCTCGAGCCTAGGATAGAGCTCCTTAAGATATTCGAAGTATACA

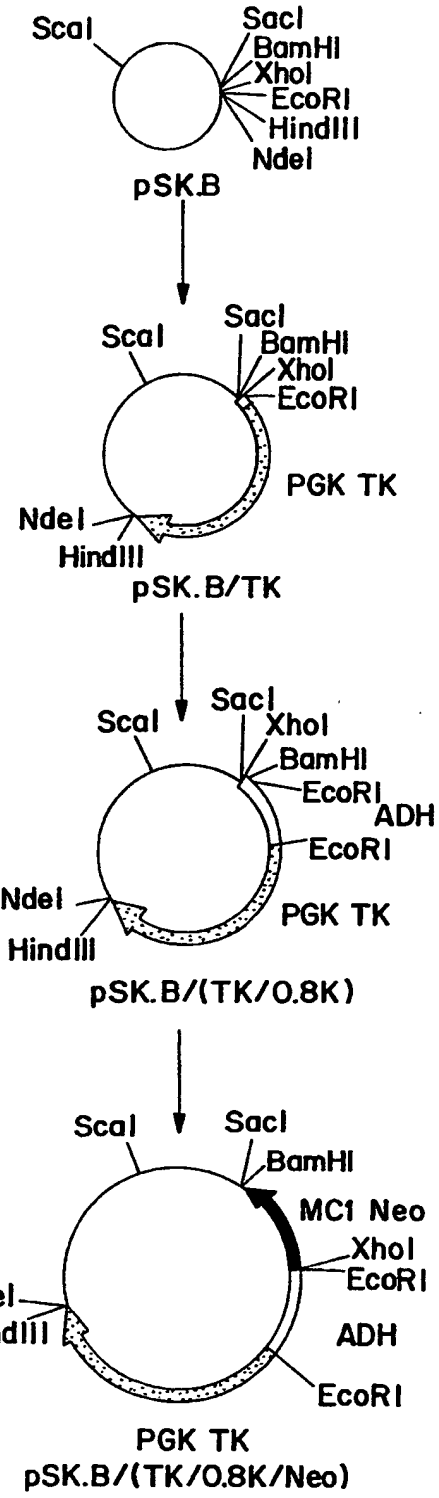
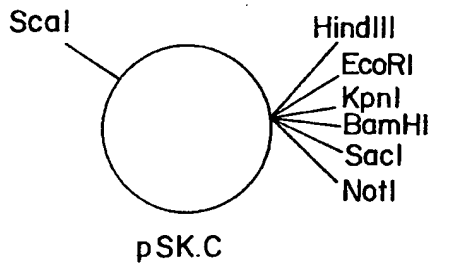
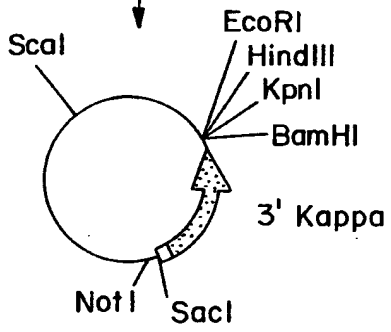


FIG.5B

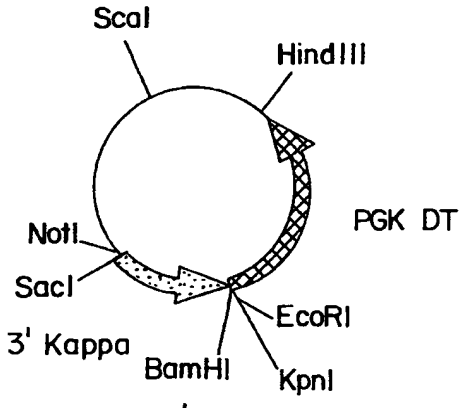
HindIII EcoRI KpnI BamHI SacI NotI
AAGCTTATAGAATTCGGTACCTCGATCCTGAGCTCATAGCGGCGCCAGCT
CATGTTCGAATATCTTAAGCCATGGAGCTAGGACTCGAGTATCGCCGCG



pSK.C



pSK.C/3'K



pSK.C/3'K/DT

FIG.5C

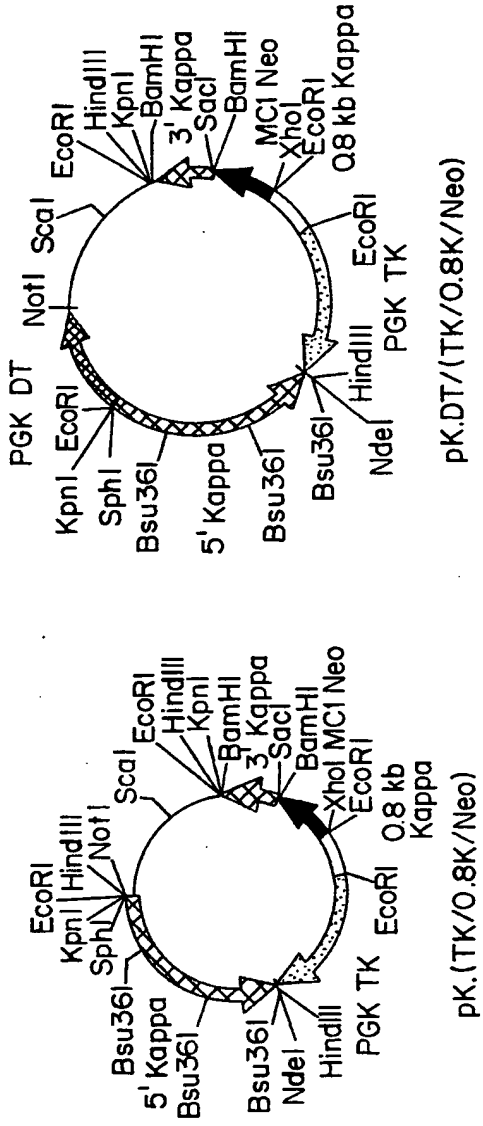
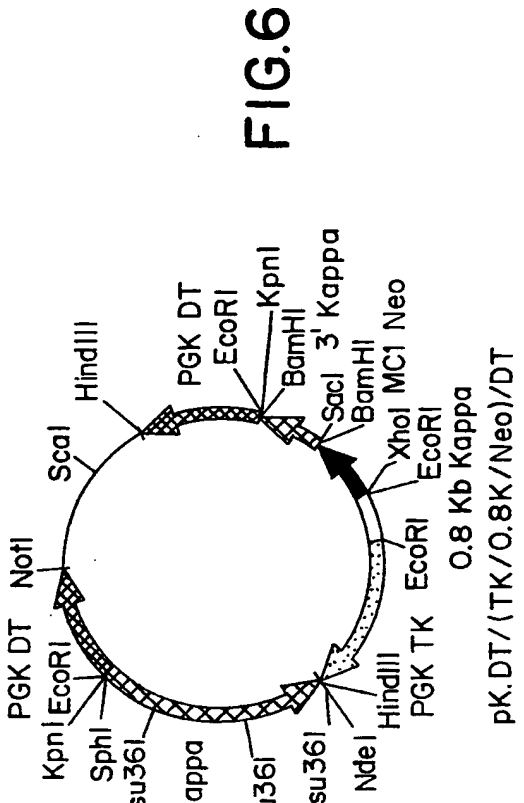

PGK DT
NotI
ScaI
EcoRI
HindIII
KpnI
BamHI
3' Kappa
SacI
BamHI
MC1 Neo
XhoI
EcoRI
0.8 kb Kappa
EcoRI
PGK TK
HindIII
NdeI
Bsu36I
Bsu36I
5' Kappa
Bsu36I
SphI
KpnI
EcoRI
pK.DT/(TK/0.8K/Neo)
EcoRI
KpnI
SphI
Bsu36I
5' Kappa
Bsu36I
Bsu36I
NdeI
HindIII
PGK TK
EcoRI
0.8 kb Kappa
XhoI
MC1 Neo
EcoRI
BamHI
3' Kappa
SacI
BamHI
EcoRI
HindIII
KpnI
ScaI
NotI
HindIII
pK.(TK/0.8K/Neo)

FIG.6


PGK DT
NotI
ScaI
HindIII
PGK DT
EcoRI
KpnI
BamHI
3' Kappa
SacI
BamHI
MC1 Neo
XhoI
EcoRI
0.8 Kb Kappa
EcoRI
PGK TK
HindIII
NdeI
Bsu36I
Bsu36I
5' Kappa
Bsu36I
SphI
KpnI
EcoRI
pK.DT/(TK/0.8K/Neo)/DT

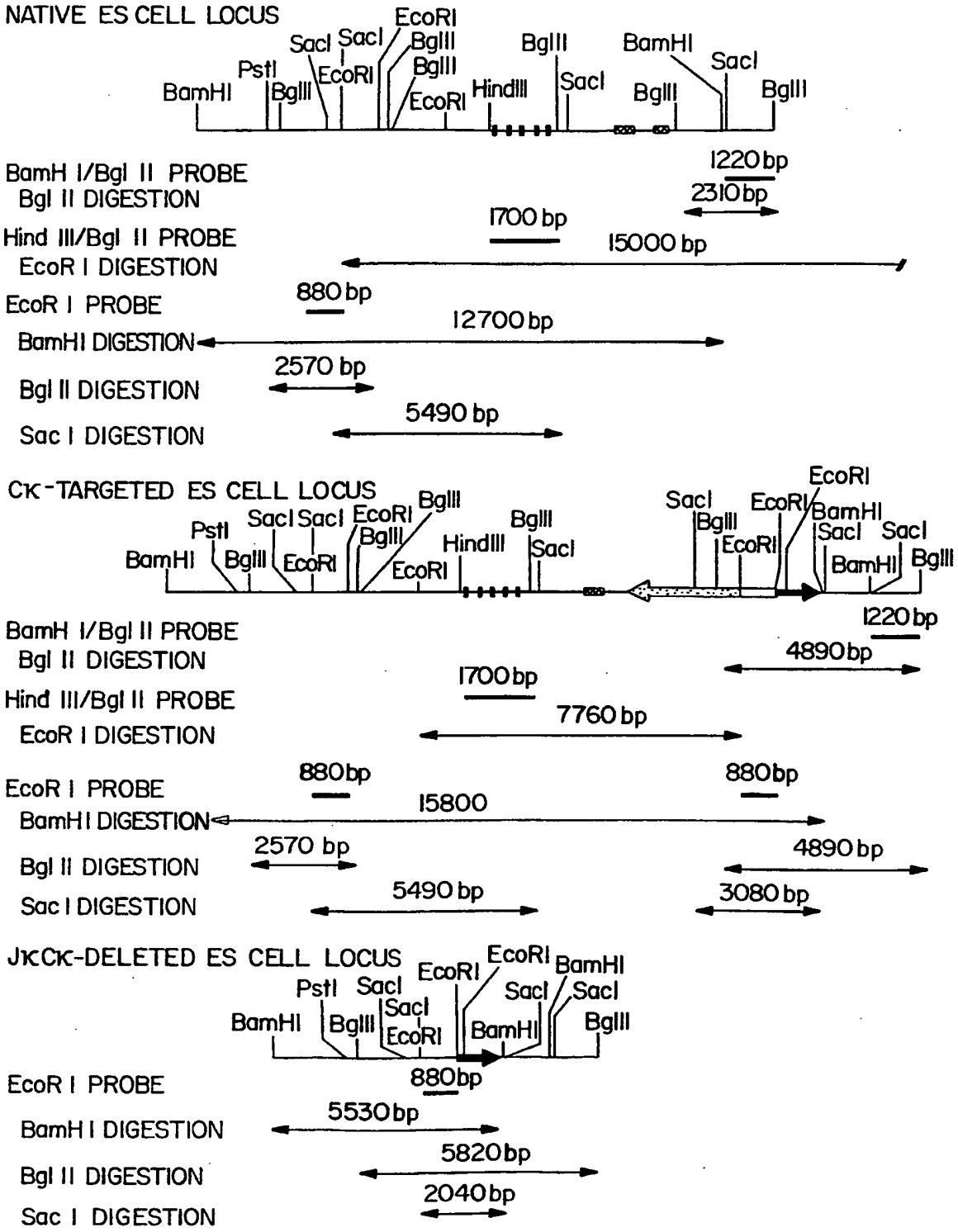

NATIVE ES CELL LOCUS
EcoRI
SacI SacI
BglII
BglII
BglII
BamHI
PstI
BglII
EcoRI
EcoRI
HindIII
SacI
BglII
BamHI
SacI
BglII
1220 bp
BamH I/Bgl II PROBE
Bgl II DIGESTION
2310 bp
1700 bp
Hind III/Bgl II PROBE
EcoR I DIGESTION
15000 bp
880 bp
EcoR I PROBE
12700 bp
BamHI DIGESTION
2570 bp
Bgl II DIGESTION
5490 bp
Sac I DIGESTION
Cκ-TARGETED ES CELL LOCUS
EcoRI
BglII
SacI
BamHI
PstI
SacI SacI
EcoRI
BglII
BglII
SacI
EcoRI
EcoRI
BamHI
SacI
SacI
BamHI
BglII
BamHI
BglII
EcoRI
HindIII
SacI
1220 bp
BamH I/Bgl II PROBE
Bgl II DIGESTION
4890 bp
1700 bp
Hind III/Bgl II PROBE
EcoR I DIGESTION
7760 bp
880 bp
880 bp
EcoR I PROBE
15800
BamHI DIGESTION
2570 bp
4890 bp
Bgl II DIGESTION
5490 bp
3080 bp
Sac I DIGESTION
JκCκ-DELETED ES CELL LOCUS
EcoRI
EcoRI
BamHI
PstI
SacI
SacI
SacI
SacI
BamHI
BglII
SacI
EcoRI
BamHI
BglII
880 bp
EcoR I PROBE
5530 bp
BamH I DIGESTION
5820 bp
Bgl II DIGESTION
2040 bp
Sac I DIGESTION

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/02676

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 15/09, 15/11, 15/13
US CL :435/172.3, 320.1; 536/23.1, 23.2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1; 536/23.1, 23.2; 935/22, 34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Human Immunology, Volume 16, issued 1986, "Molecular Analysis of HLA Class I and Class II Antigen Loss Mutants Reveals a Homozygous Deletion of the DR, DQ, and Part of the DP Region: Implications for Class II Gene Order", pages 205-219, see entire article.	29 --- 27, 28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 JUNE 1994	Date of mailing of the international search report JUL 05 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer George C. Elliott Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS/DIALOG/INTELLIGENETICS
search terms: sequences 1-12, homologous recombination, immunoglobulin genes, MHC, HLA, targeted, inactivation, deletion, intramolecular recombination